

MEDICAGO FALCATA L. IN ESTONIA: CHROMOSOMAL AND MORPHOLOGICAL VARIABILITY, DISTRIBUTION AND VULNERABILITY OF TAXA

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The morphological and chromosomal variation of *Medicago falcata* L. was studied in 47 populations in Estonia to ascertain differences between two chromosome races: diploids ($2n=16$) and tetraploids ($2n=32$). The results showed that both chromosome races are growing in Estonia but they are not well distinguishable on the basis of morphological characters only. Due to intensive introgression in the *M. falcata/sativa* complex the number of pure natural *M. falcata* populations is diminishing in Estonia as they are under pressure and vulnerable due to intensive crossing with the genetic material of very different origin.

Key words: *Medicago falcata* L., chromosome races, *Medicago falcata/sativa* complex, introgression, morphology

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Introduction

Sickle medics (*Medicago falcata* L.) are agriculturally important legumes (*Fabaceae*) that were cultivated already long ago (Bender & Aavola 1999). *Medicago falcata* is characterized by yellow flowers and sickle-shaped pods (Talts 1959). Sickle medic has two chromosome races: diploid ($2n=16$) and tetraploid ($2n=32$) (Lesins & Lesins 1979).

Medicago falcata belongs, together with *M. sativa* L. and their hybrid (*M x varia* Martyn), to the *M. falcata/sativa* complex (Quiros & Bauchan 1988). *Medicago sativa* is characterized by purple flowers and coiled pods. *Medicago x varia* shows intermediate characteristics: flower colour is

variegated (blue, yellow, brown, white), but pods are also coiled (Small & Brooks 1984).

Medicago falcata is natural in West- and North-Estonia on calcareous soils (Talts 1959), while *M. sativa* was introduced to Estonia only at the turn of the 18th and 19th centuries (Bender & Tamm 1998). It prefers more fertile soil (Rufener All Mazyad & Ammann 1998). Their hybrid, *M x varia*, is also cultivated in Estonia. All three taxa have been used successfully in plant breeding in Estonia. Breeding material has been brought also from southern regions and even from North-America (Bender & Aavola 1999). *M. sativa* and *M x varia* run wild easily and are both naturalized in Estonia (Kukk & Kull 2005, Kukk 1999). Tetraploid *M. falcata*, *M x varia* and *M. sativa*

mate freely under natural conditions, producing fertile hybrids. In the *M. falcata* /*sativa* complex introgression occurs from cultivated plants to natural populations and hybrids between weeds and crops are frequent (Small 1984).

There are known two classifications of the genus *Medicago* L. In Estonia, up to now, we have followed the classification of the famous Russian taxonomist Grossheim. His classification is based on the morphological species concept. *Medicago sativa*, *M. x varia* and *M. falcata* are recognized as independent species. This classification also separates *M. borealis* (2n=16) and *M. romanica* (2n=16, 32) as independent species (Grossheim 1945).

Nowadays, the American taxonomists K. and I. Lesins, relying on the biological species concept, support sickle medic as a subspecies – *Medicago sativa* ssp *falcata*. Therefore, *M. falcata* and *M. x varia* are classified as subspecies of *M. sativa* while *M. borealis* and *M. romanica* are considered as synonyms of *M. falcata*. It is only specified that *M. falcata* has two chromosomal races (Lesins & Lesins 1979, Quiros & Bauchan 1988). This approach seems to gain more popularity also in Europe.

However, the morphological variability of the chromosomal races of *M. falcata* has not been analysed sufficiently: in *M. falcata* only pollen diameter has been used to discriminate between tetraploids and diploids, while in *M. sativa* s.l.

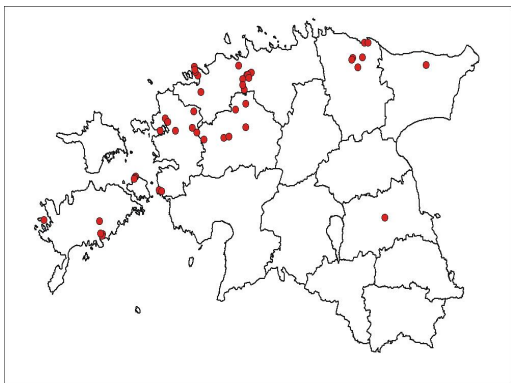


Fig. 1. Sampling sites of the studied populations

the diploids have been recognized as a separate taxon.

Aims of the study

1. Do both diploid and tetraploid *Medicago falcata* L. races grow in Estonia and what are their distribution patterns like?
2. Are the diploid and tetraploid races of *Medicago falcata* L. morphologically distinguishable?
3. How large is the extent of morphological discontinuity and chromosomal numbers between populations from different ecological conditions?
4. Has the distribution area of pure *Medicago falcata* L. diminished?

Material and methods

Plant material

The morphological variation of *Medicago falcata* was studied on the basis of 208 plant specimens collected in 2005 and 2006 from 47 populations located mostly in North- and West-Estonia (Figure 1). From each population 1-9 plants were collected, from each plant 1-5 shoots were measured. Altogether 435 shoots were studied.

For chromosome counts seeds were gathered from the same populations, yet not from all of them. In 2005 seeds were collected from 5 populations and in 2006 from 18 populations.

Morphometric measurements were made on the air dry herbarium material of mature specimens. For each plant, 34 different morphological characters from different shoots were counted or measured (in mm) using a ruler and a stereomicroscope Olympos SZ/SC (Table 1). Hairiness was estimated using magnification 1x20; length of the serrated leaflet, length and width of the topmost tooth, length and width of the calyx and the calyx tooth were measured using magnification 1x10. The relief of the veins was estimated at 1x10. The following ratio characters were also used: ratio of leaflet length to leaflet width, ratio of raceme length to number of flowers, and ratio of leaflet length to number

Table 1. Morphological characters measured

Character	Notation
1.Length	LENG
2.Diameter of the stem	DSTE
3.Hairiness of the stem	HASTEM
4.Length of the stipule	LSTIP
5.Width of the stipule	WSTIP
6.Length of the lower leaflet	LLLEAF
7.Width of the lower leaflet	WLLEAF
8.Length of the lower petiole	LLPET
9.Hairiness on the upper side of the lower leaflet	HULLEAF
10.Hairiness on the lower side of the lower leaflet	HLLLEAF
11.Number of teeth of the lower leaflet	NTLLEAF
12.Length of serrated part of the lower leaflet	LSLLEAF
13.Length of the topmost tooth of the lower leaflet	LJLLEAF
14.Width of the topmost tooth of the lower leaflet	WJLLEAF
15.Length of the upper leaflet	LULEAF
16.Width of the upper leaflet	WULEAF
17.Length of the upper petiole	LUPET
18.Hairiness on the upper side of the upper leaflet	HUULEAF
19.Hairiness on the lower side of the upper leaflet	HLULEAF
20.Number of teeth of the upper leaflet	NTULEAF
21.Length of serrated part of the upper leaflet	LSULEAF
22.Length of the topmost tooth of upper leaflet	LJULEAF
23.Width of the topmost tooth of the upper leaflet	WJULEAF
24.Relief of veins (unraised, average, strongly raised)	REVEIN
25.Number of veins	NUVEIN
26.Length of the raceme	LRACE
27.Number on flowers in the raceme	NFRACE
28.Length of the pedicel	LPEDIC
29.Length of the flower	LFLOW
30.Length of the calyx	LCALYX
31.Width of the calyx	WCALYX
32.Hairiness of the calyx	HCALYX
33.Length of the calyx tooth	LCALYCT
34.Width of the calyx tooth	WCALYXT
35.Length of the raceme/number on flowers in the raceme	LRACE/NFRACE
36.Length of the upper leaflet/number of veins	LULEAF/NUVEIN

of veins. Leaflet characters were measured for the leaves situated at the basis and in the central part of the shoot.

The material collected is preserved in the Herbarium of the Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences (TAA).

Data processing

Cluster analysis was performed with the program package SAS 8.01. Cluster analysis was used to study the pattern of formation of groups. For cluster analysis Ward's clustering method with the semi-partial R-square distance was employed to measure similarities between clusters.

The formation of groups was studied also with principal component analysis (PCA). Principal component analysis was made on the basis of

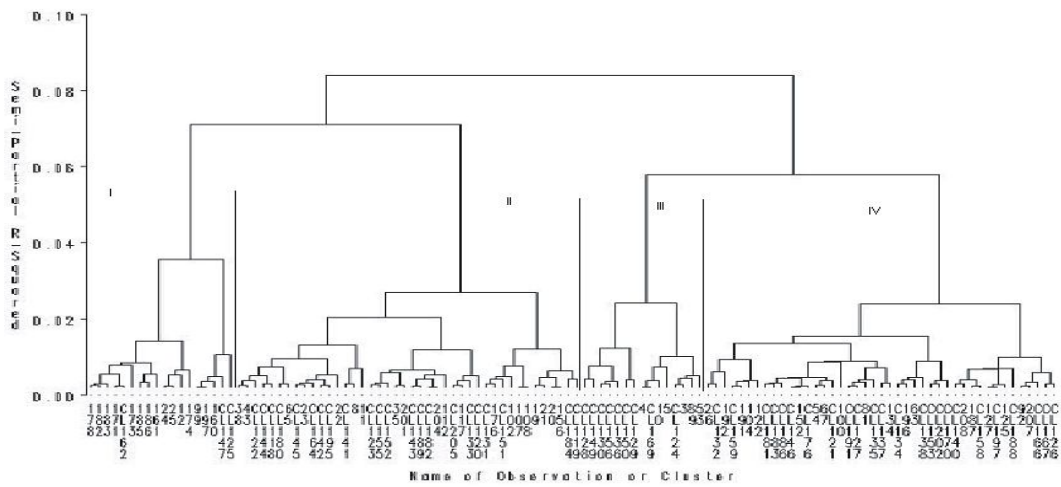


Fig. 2. Dendrogram of *M. falcata* (Ward's algorithm), measured characters

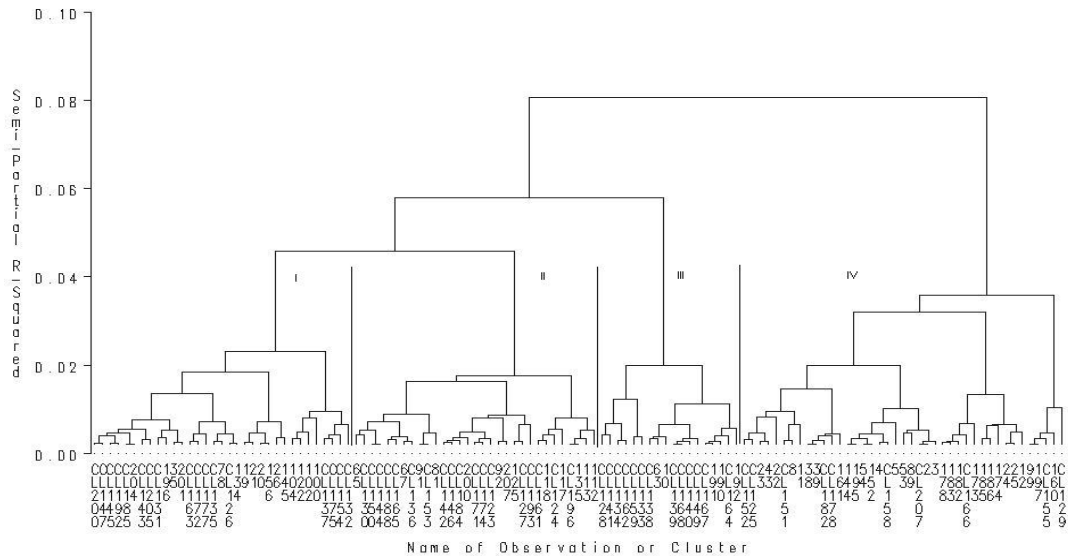


Fig. 3. Dendrogram of *M. falcata* (Ward's algorithm), measured and ratio characters

the groups obtained with cluster analysis. The generalized linear model (GLM) was used to find out significantly different characters between the groups.

For data processing the average values of morphological characters were calculated for each plant.

Chromosome counts

Chromosome counts were made from the root tips of seedlings using a light microscope (Zeiss Standard 20) at magnification 10x100. Photos were taken with a camera Canon EOS 300D DIGITAL. For breaking hardseediness, seeds were scarified manually with sandpaper. The seeds were germinated in Petri dishes on moistened filter paper for 5-7 days at room temperature. For chromosome slides, radicals with a length of 1-2 cm were used.

Table 2. Significant differences between the groups (measured characters)

Character	I-II	I-III	I-IV	II-III	II-IV	III-IV
LENG	X	X	X		X	
DSTE	X	X	X		X	X
HASTEM	X		X	X	X	X
LSTIP	X	X	X	X	X	X
WSTIP	X		X		X	X
LLPET	X	X	X			
LUPET			X			
LLLEAF	X	X	X	X	X	X
WLLEAF	X	X	X		X	
LUPET	X		X		X	X
WULEAF			X	X	X	X
NTULEAF			X		X	X
LSLLEAF	X	X	X		X	X
HULLEAF			X		X	X
HLLLEAF		X	X	X	X	X
HUULEAF	X	X	X		X	X
HLULEAF	X	X	X	X	X	X
LPEDIC	X		X		X	X
LRACE		X	X		X	X
NFRACE	X	X	X		X	X
LFLOW			X		X	X
LCALYCT	X	X	X		X	X
WCALYXT					X	
LCALYX	X		X		X	X
HCALYX	X	X	X			
REVEIN	X	X				X

Slides were made in the morning (9.00-10.00) when cell division is the most intensive (Mujeeb-Kazi & Miranda 1985). It is important to accumulate metaphase chromosomes, which are condensed and dispersed and thereby better countable (Laarman 1997). For accumulating metaphases, hydroxyquinoline (Wantabe *et al* 1990) or hydroxyurea (inhibitors of DNA syntesis) and trifluralin (metaphase blocking agent, also synchronizes phases of cell division) were used (Lee *et al* 2000, Lee *et al* 1996). Before treatment with trifluralin, the seedlings were rinsed with cold water. This blocks the influence of hydroxyurea and also helps accumulate metaphases (Lee *et al* 1996). For staining chromosomes, aceto-orceine or lactopropionic orceine were employed (Armstrong 1995). For macerating tissues, radicles were hydrolysed with 1:1 HCl water solution or heated in 45% acetic acid using a spirit lamp. Before staining, the root

tips were fixed in Farmer's fixative (1:3 glacial acetic to absolute ethanol). After staining, with a sharp razor, 1-2 mm tips were cut from the root and macerated. The root tips were squashed in 45 % acetic acid or in glacial acetic and glycerin (1:1). Finally, the slides were pressed plane.

Results

Cluster analysis

The dendrograms obtained with cluster analysis are branched into four clusters (Figure 2, 3). The lower part of the dendrogram has been removed and replaced with numbers referring to a plant or a cluster.

When the measured characters were only used, the groups differed in the values of 26 characters (Table 2). When considering also the ratio characters, the groups differed in the values of 24 characters (Table 3). The following Tables include only the characters which are significantly different in at least one pair of the groups studied. Significant differences are marked with X (p-level < 0.05).

According to the measured characters, groups I and IV are the most different. Groups III and IV differ in 22 characters, groups II and IV differ in 18 characters, and groups I and III differ in 15 characters. The most similar groups are II and III with only 7 significantly different values of morphological characters (Table 2).

When also the ratios of the character values are considered, dissimilarities are the following:

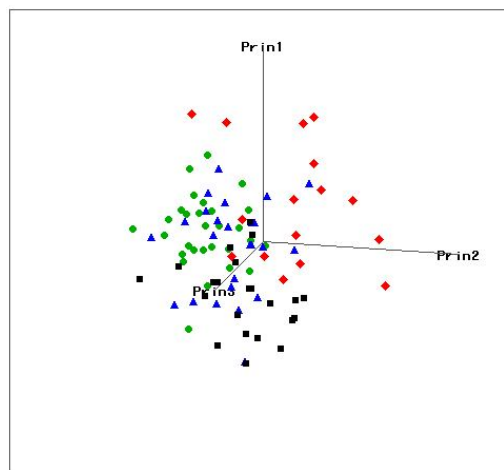
Table 3. Significant differences between the groups (measured and ratio characters)

Character	I-II	I-III	I-IV	II-III	II-IV	III-IV	
LENG	X	X	X	X	X	X	The plants belonging to group IV are the largest. The plants are the highest, the diameter of the stem is the largest and their leaflets and stipules are the widest. The plants are the hairiest and the leaflets have the highest number of teeth. The length of the petiole and the length of the raceme are the largest. The number of flowers in the raceme is the highest. The flowers are the longest, with the longest calyx and the calyx jag. To group IV belong 28 specimens, five of them sharing characteristics with <i>M. x varia</i> .
HASTEM	X	X	X	X	X	X	
LSTIP	X	X					
WSTIP	X	X	X	X	X	X	
LUPET			X		X	X	
LLLEAF	X	X		X		X	
WLLEAF		X		X		X	
LULEAF			X		X	X	
WULEAF		X	X		X	X	
HULLEAF	X		X	X		X	
HILLEAF	X	X	X	X	X	X	
HUULEAF	X	X	X				
HLULEAF			X		X	X	
LPEDIC	X	X	X				
LRACE			X	X		X	
NFRACE		X		X		X	
LCALYCT		X		X		X	
LCALYX			X		X	X	
WCALYX			X			X	
HCALYX	X		X	X		X	
REVEIN	X						
LRACE/NFRACE		X	X	X	X		
LULEAF/NUVEIN		X			X		

groups III and IV in 18 characters, groups I and IV in 17 characters, groups I and III in 14 characters, and groups II and III as well as groups I and II in 12 characters. The most similar are groups II and IV with 11 significantly different values of morphological characters (Table 3).

To characterize the groups, the mean values of their morphological characters, including the ratio characters, were calculated. Only statistically significantly different values of the morphological characters were considered. Among the ratio characters only the ratio of raceme length to number of flowers and the ratio of leaflet length to number of veins appeared statistically significant.

As the groups are better distinguishable with the use of measured characters only, their characterization is based on the values of the measured characters.



- ◆ - group I
- ▲ - group II
- - group III
- - group IV

Fig. 4. Ordination of clusters obtained by principal component analysis (measured and ratio characters)

The group most different from group IV is group I. The plants in this group are the smallest with the thinnest stems, the stipules and leaflets are the narrowest and shortest, as are also the petioles. The hairiness of the leaflets occupies the second place among the groups. The number of teeth on the leaflet is the smallest. The racemes are short, with the smallest number of flowers. The calyx is narrow and less hairy. The relief of the veins is similar to that in groups I and IV. The veins are less raised compared to those in groups II and III. To group I belong 31 specimens. Only one of them shares characteristics with *M. x varia*.

Groups II and III are the most similar. Significantly different characters for these groups are hairiness of the stem and leaflets, length of the stipule and the leaflet and width of the leaflet. The plants in group III have hairier stems, longer stipules and leaflets, the leaflets are also narrower and less hairy. The plants in groups II and III form an intermediate group between clusters I and IV,

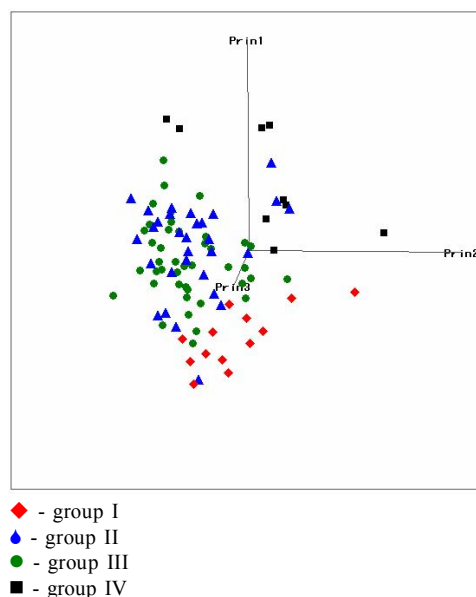


Figure 5. Ordination of clusters obtained by principal component analysis (measured characters)

Table 4. Correlations between the main components and the characters (measured characters)

Character	Main component I	Main component II	Main component III
DSTE	0.256991	0.120837	0.089950
HASTEM	0.129346	0.122532	0.090159
LSTIP	0.255219	0.103970	0.014159
WSTIP	0.231166	0.061584	0.032299
LLPET	0.230361	0.161229	0.0194982
LLLEAF	0.297203	0.185540	0.033523
WLLEAF	0.284255	0.134886	0.149000
LULEAF	0.284470	0.139133	0.186539
WULEAF	0.218269	0.262341	0.234704
WJLLEAF	0.215211	0.125588	0.047798
HULLEAF	0.063983	0.311554	0.216885
HLLLEAF	0.040531	0.351454	0.095412
HUULEAF	0.073312	0.420512	0.016077
HLULEAF	0.052523	0.346281	0.111982
LPEDIC	0.110250	0.074255	0.321803
LRACE	0.133356	0.014340	0.298376
NFRACE	0.161140	0.037614	0.181436
LFLOW	0.096532	0.104131	0.239772
LCALYCT	0.181988	0.014451	0.318210
WCALYXT	0.104793	0.048022	0.295313
LCALYX	0.215992	0.080999	0.270263

which is based on the size of the plants (length, diameter of the stem, length and width of leaflets and stipules) and on the characteristics of the racemes and flowers (length of racemes and flowers, number of flowers, length of the calyx and calyx tooth). Groups II and III both consist of 74 specimens. Three plants in group II and one plant in group III share characteristics with *M. x varia*.

Still, many values of the morphological characters are not significantly different for different groups. Also, several plants collected from the same sample places are clustered into different groups. As it is the case with morphological characters, which are overlapping between the groups to some extent, it appeared that the clusters were not formed according to the ecological conditions in which the plants were growing.

During this study, in most of the populations, in addition to yellow-flowered plants, also some plants with hybrid flower colours were found. Only 7 populations consisted of purely yellow-flowered plants. For these populations it was

characteristic that they occurred in areas separated from crop fields (Kuusiku, Rõuma, Pakri bank), or were very small (Põdruse, Letipea cape, Laelatu), or were growing in extremely dry conditions (Paldiski northern port) (Figure 8).

Principal component analysis

Principal component analysis was performed using the groups formed in cluster analysis. The

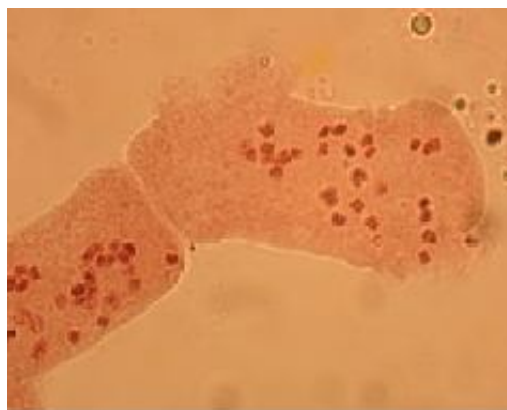


Fig. 6. Chromosome number: $2n=32$. Sampling site: Paldiski. Photo: Karin Kaljund

Table 5. Correlations between the main components and the characters (measured and ratio characters)

Character	Main component I	Main component II	Main component III
DSTE	0.227355	0.171340	0.058190
LSTIP	0.232288	0.138994	0.007700
WSTIP	0.217786	0.007634	0.005566
LLPET	0.228302	0.098952	0.232734
LLLEAF	0.272961	0.246723	0.012552
WLLEAF	0.278455	0.099119	0.218897
LULEAF	0.294780	0.148154	0.078146
WULEAF	0.234024	0.314907	0.089012
HULLEAF	0.053169	0.206662	0.296053
HLLLEAF	0.033222	0.270219	0.175113
HUULEAF	0.072972	0.360394	0.158077
HLULEAF	0.037072	0.253765	0.200334
LPEDIC	0.087746	0.146132	0.310197
LFLOW	0.103325	0.097065	0.285592
NFRACE	0.141195	0.024016	0.203801
LCALYCT	0.144526	0.093217	0.312592
WCALYXT	0.074633	0.115720	0.272076
LCALYX	0.185798	0.016322	0.298110
LLLEAF/WLLEAF	0.014842	0.269560	0.036532
LULEAF/WULEAF	0.213372	0.144039	0.039439

results are described by three main components. Considering only the measured characters main components describe only 38% of dispersal of the characters' values. When also the ratios' values were included the main components described even less – only 36% of the dispersal of the characters' values. It means that as the main components describe a very small part of the dispersal of the characters' values, distinct groups have not emerged in the figures (Figure 4, 5).

Correlations between the main components and the characters are shown in Tables 4 and 5. The strongest correlations are presented in bold.

It can be summarized that the first main component is more strongly correlated with the characteristics, which describe the size of plants; the second main component is more strongly correlated with hairiness and the third main component is more strongly correlated with the characteristics of the racemes and flowers.

Correlations between the main components and the values of the characteristics are weak and evident associations are lacking.

However, principal component analyses yielded the same result as cluster analysis: the most different are groups I and IV and the most similar are groups II and III.



Figure 7. Chromosome number: $2n=16$. Sampling site: Kunda. Photo: Karin Kaljund

Chromosome numbers

The ploidy level of the studied sprouts was tetraploid ($2n=32$) (Figure 6). However, in 2005 a few diploid offspring were found in three populations in North-Estonia (Paldiski northern port, Pakri bank, Kunda) (Figures 7,8). Presumably, these are mixed populations. However, in the year 2006 only tetraploid numbers were counted in the same populations. No triploid chromosome counts were made, either.

In cluster analysis, the plants from these mixed populations were classified as belonging to groups I, II and III.

In this study different methods were used for preparing slides. The best results were received with hydroxyurea and trifluralin. This treatment allowed to accumulate more metaphases than treatment with hydroxyquinoline. The chromosomes were stained more strongly by using lactopropionic orceine compared with aceto-orceine.

Discussion

The studied populations appeared to be mixed populations, and as the chromosome numbers were determined from the seed samples which were collected from the whole population (not from single plants), relationships between the morphological characters and the ploidy level of the plants could not be clarified.

Cluster analysis discriminated the plant specimens by size. Polyploids are characterized by larger cells (Ramsey & Schemske 1998), however, for recognizing diploids and tetraploids, also the size characters of plants have been used (Borill & Lindner 1971, Ramsey & Schemske 1998). In the present study, plants from mixed populations belonged, according to the results of the cluster analysis, to groups I, II and III. These groups consist of smaller plants compared to plants in group IV and their morphological characters are the most similar. However, when

comparing diploid and tetraploid plants of *M. falcata*, Small (1985) has found, that the values of the morphological characters of tetraploids were not significantly higher than those of the diploids. The morphological characters of the tetraploid and diploid *M. falcata* are to some extent overlapping (Evans 1955, Small 1985). According to Small (1985), diploid plants have less hairy leaflets and the number of leaflet teeth is smaller, the calyx is longer, the petals are wider, the veins are less raised, the pods are less coiled and also hairier and the seeds are smaller. The length and width of the leaflets, the length of the pedicel and the number of flowers in the raceme appeared to be insignificant characters for distinguishing diploids and tetraploids. Presumably, diploid *M. falcata* is less variable than tetraploid *M. falcata* (Lesins & Lesins 1979). Blue-flowered diploid *M. sativa* ssp. *carulea* is even less variable than diploid *M. falcata* (Small 1985).

As the results of this study showed, the leaflets of specimens in groups I, II and III are the hairiest with the smallest number of teeth. The veins are less raised in groups I and IV. However, group IV consists of only tetraploid plants. The length of the calyx is smallest in groups I, II and III. Considering that most of the plants in groups I, II and III are tetraploids, it can not be claimed on the basis of the current results that these morphological characters are the best for distinguishing between tetraploids and diploids. It can be concluded that the morphological characters of diploids and tetraploids are somewhat overlapping.

To find significantly different morphological characters for distinguishing between the diploids and the tetraploids, it would be necessary to grow plants from seeds with the already determined ploidy level. For a comparative study the work should be carried out in similar environmental conditions to avoid the influence of ecological conditions.

Several studied plants growing in one and the same population in similar ecological conditions were clustered into separate groups. As it was

the case with morphological characters, which are overlapping to some extent between the groups and can not separate clusters, it appeared that clusters were not formed, either, according to the ecological conditions in which the plants were growing.

The studied populations did not grow in very different ecological conditions. Mostly, they occupied sunny sites on roadsides, meadows and along the coastline. Hence it can be concluded that the morphological characters of the populations of *M. falcata* are quite variable.

In the course of this study it was found that both diploid and tetraploid *M. falcata* races are present in Estonia. Among the populations studied 15 were tetraploid populations and three were mixed populations. Populations with only diploid plants were not found. The results of earlier studies have indicated the occurrence of mixed populations in Estonia (Bender & Tamm 1998). Diploid and tetraploid plants of *M. falcata* can grow in mixed (Small & Bauchan 1984) or separate populations (Rufener All Mazyad & Ammann 1998), as the earlier studies in the world have shown. However, in regions where both diploids and tetraploids occur, tetraploids have a more extensive distribution area (Lesins & Lesins 1979). The results of the current study support this argument.

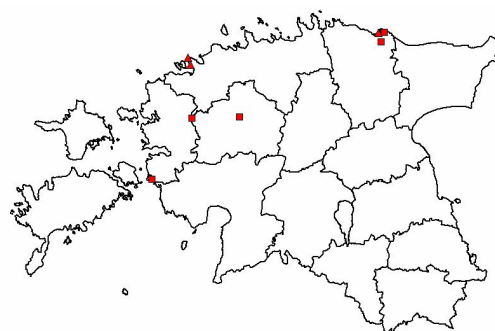


Figure 8. Sampling sites: ■ - "pure" populations, ◆ - populations consisting of diploid *M. falcata*

In the *Medicago sativa/falcata* complex, introgression from crop fields to natural populations is intensive and hybrids between weeds and cultivated plants are often found (Rufener All Mazyad & Ammann 1998). Necessary conditions for gene flow from crops to weeds are overlapping distribution areas and the same flowering times and pollinators (Jenczevski *et al* 1999). In Estonia both of these conditions are fulfilled; the most important pollinators are bumble bees (Karise 2003) which can cause cross-pollination at a distance of up to 1600 metres (Bradner 1965).

Most of the studied populations were situated in areas with strong human impact and in most of these populations there occurred also hybrid plants. Only 7 populations consisted of purely yellow-flowered plants. For these populations, it was characteristic that they were located in areas separated from crop fields (Kuusiku, Rõuma, Pakri bank), were very small (Põdruse, Letipea cape, Laelatu) or were growing in extremely dry conditions (Paldiski northern part). It is known that plants of hybrid origin are better adapted to growing in habitats with a strong impact of human activity compared to natural habitats (Small 1984). As natural plants are important breeding material in plant breeding, also introgression from weeds to cultivated plants takes place. Reciprocal introgression involves morphological characters and habitat requirements, which are not any more strictly different for weeds and crops. Cultivated plants are able to grow in natural habitats and can easily run wild under natural conditions (Small 1984). In Estonia, *M. x varia* is well adapted to natural conditions. *Medicago x varia* can also, owing to its vigorous growth, become dominating over other species (Estonian database of alien species 2003).

In Estonia, natural populations of *M. falcata* have a hybrid character to some extent due to introgression between cultivated and wild growing plants (Adojaan & Jaagus 1977). Over the years ample seed material has been introduced into Estonia from different regions of

the world by plant breeders (Bender & Aavola 1999). Therefore, it can be concluded that „pure“ natural populations of *M. falcata* in Estonia are under pressure and vulnerable due to introgression and crossing with genetic material of very different origin.

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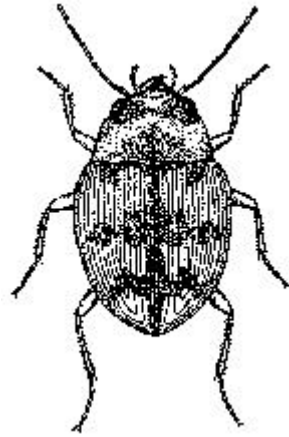
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